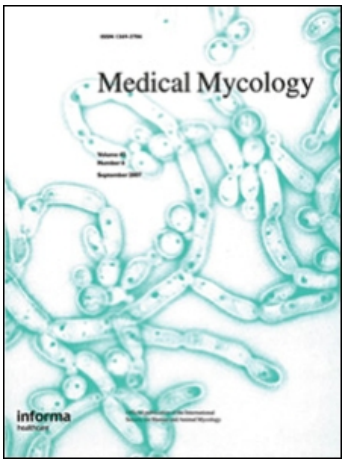

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Effect of competition on the production and activity of secondary metabolites in *Aspergillus* species

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Secondary metabolites are of intense interest to humans due to their pharmaceutical and/or toxic properties. Also, these metabolites are clinically relevant because of their importance in fungal pathogenesis. *Aspergillus* species secrete secondary metabolites when grown individually and in the presence of other fungal species. However, it is not known whether secreted secondary metabolites provide a competitive advantage over other fungal species, or whether competition has any effect on the production of those metabolites. Here, we have performed co-cultivation competition assays among different species of *Aspergillus* to determine relative species fitness in culture, and to analyze the presence of possible antifungal activity of secondary metabolites in extracts. The results show that, for the most part, at 30°C only one species is able to survive direct competition with a second species. In contrast, survival of both competitors was often observed at 37°C. Consistent with these observations, antifungal activity of extracts from cultures grown at 30°C was greater than that of extract from cultures at 37°C. Interestingly, culture extracts from all species studied had some degree of antifungal activity, but in general, the extracts had greater antifungal activity when species were grown in the presence of a competitor. Using gas chromatography it was determined that the composition of extracts changed due to competition and a shift in temperature. These findings indicate that co-cultivation could be a very promising method for inducing and characterizing novel antifungal compounds produced by species of *Aspergillus*.

Keywords *Aspergillus*, secondary metabolites, antifungal activity

Introduction

Aspergilli are opportunistic fungal pathogens in immunocompromised individuals most commonly associated with diseases of the respiratory tract, such as invasive aspergillosis and fungal sinusitis, but can also invade and cause disease in the circulatory, central nervous, cutaneous, gastrointestinal and genitourinary systems [1]. Each species of *Aspergillus* can produce a range of secondary metabolites associated with fungal growth and development [2,3]. In some cases these metabolites have been implicated in disease, since they appear to be virulence factors [3–7]. Interestingly, many of these secondary metabolites have been used in medicine for their antiviral, antibacterial, tumor suppressing, antihypercholesterolemic and immunosuppressant activities [8]. However, some of these metabolites, called mycotoxins [9], are toxic to the host , for example, aflatoxin is one of the most toxic and powerful carcinogens known.

The mechanisms regulating production of secondary metabolites are not well understood. It has been shown that several environmental variables have an impact on their production: pH below 4.0 seems to enhance the

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production of aflatoxin and sterigmatocystin [10]; both carbon and nitrogen sources, as well as zinc availability, affect aflatoxin production [11,12]; and plant or fungus-derived linoleic acid can also modulate the production of aflatoxins [13–16]. Environmental signals may be perceived and relayed in the cytosol to the protein LaeA, which appears to govern the expression of gene clusters responsible for producing most secondary metabolites [17]. Interestingly, a Δ*laeA Aspergillus fumigatus* strain has reduced virulence, which, at least in part, results from decreased levels of secreted secondary metabolites [18]. A recent study demonstrated that a strain of *A. fumigatus* incapable of producing gliotoxin, another secondary metabolite, was considerably less virulent in a murine model [19], underscoring the importance of secondary metabolites in disease.

Apart from environmental signals, competition between different fungal species also modulates production of secondary metabolites. For instance, aflatoxin production by *Aspergillus flavus* is affected by competition with other Aspergilli [20–23], and production of secondary metabolites by other fungi, such as *Microdochium* and *Fusarium* spp., is modified due to the presence of competitors [21,24–26]. Fungi compete in various ways, including: (i) rapid growth, sporulation or stress recovery; (ii) use of inhibitors against other species; (iii) negation of inhibitors; and, (iv) the colonization of a special niche [27]. However, it is not clear whether any of the secondary metabolites produced by Aspergilli provide a competitive advantage against other fungi by acting in any of these manners. Similarly, it is not known whether competition has any effect on the production of other secondary metabolites beside aflatoxin, or lastly whether competition among different species of *Aspergillus* results in variable secondary metabolite production. We hypothesized that by inducing competition among Aspergilli, it would be possible to exploit the natural mechanisms for inducing previously undetected secondary metabolites, which might have antifungal properties, or otherwise provide a competitive advantage. In this study we investigated the effect of interspecies co-cultivation competition among Aspergilli on the production of secondary metabolites. We were especially interested in determining whether competition led one or both species to produce metabolites that could induce rapid sporulation (a sign of fungal stress), inhibit growth of other species, or suppress inhibition by other species, as these would be potential therapeutic agents to be developed further. We found that many culture extracts exhibited antifungal activities, and that in many cases competition between species induced the production of even more antifungal compounds.

Materials and methods

Strains and culture conditions

The strains used in this study are listed in Table 1. Strains were routinely grown on Yeast Dextrose Agar (YDA, yeast Extract 5.0 g, dextrose 20.0 g, agar 10.0 g, water to 1 l) at either 30°C or 37°C, unless stated otherwise.

Extraction of secondary metabolites

The agar in the culture plates was cut into small pieces and transferred to a 500-ml Erlenmeyer flask. Forty-ml of acetone were added and the flask was shaken covered for 1 h at room temperature. Forty-ml of chloroform were then added and shaken for an additional hour. Roughly 70 ml from the organic solvent phase were transferred to a clean, autoclaved beaker and allowed to evaporate in the fume hood overnight. The precipitate was suspended in 1 ml of acetone and stored in a sealed glass vial.

Assay for antifungal activity in extracts

The entire 1 ml extract from one plate in acetone was spread on a YDA plate and allowed to air dry for 1 h. Five different fungal species were point inoculated on the plate using sterile toothpicks and incubated for 4 days at 30°C. Growth was compared to that on YDA media covered with 1 ml pure acetone alone as controls. Antifungal activity was assayed quantitatively by observing any reduction in growth compared to the controls, as determined by colony diameter measurements at 36 hours. Drastically reduced growth (DRG) corresponded to less than 30% of the control growth, reduced growth (RG) 31–65% of control growth,

Table 1 Strains used in this study.

Strain	Reference
Aspergillus clavatus NRRL1	[43]
Aspergillus fisheranus (teleomorph Neosartoryo *fischeri*) NRRL181	[43]
Aspergillus flavus NRRL52237	[44]
Aspergillus fumigatus Af293	[43]
Aspergillus fumigatus FGSC1163	[43]
Aspergillus nidulans (teleomorph *Emericella nidulans*) FGSCA4	[45]
Aspergillus niger NRRL328	[46]
Aspergillus niger NRRL3122	[47]
Aspergillus oryzae RIB40	[48]
Aspergillus terreus NIH2426	Broad Institute

slightly reduced growth (SRG) 66–92% of control growth and control (C), 93–100% control growth.

Co-cultivation competition analysis

Equal numbers of conidia (10^7) of two fungal strains were premixed in 50 µl of 0.05% Triton X-100 and plated on YDA. Strains were allowed to grow together at 30°C or 37°C for 4 days, at which point spores and hyphae were collected, serially diluted, plated on fresh YDA, and incubated at 30°C. Differences in colony morphology and color were observed after 24 h or 48 h, and the amounts of colonies relative to one another were noted. All species and strains utilized in this study are readily distinguishable by colony color and morphology on YDA plates at 30°C (see Fig. 1).

Chromatography

The extracts were dissolved in 0.5 ml methanol, filtered and analyzed by HLPC-DAD [28]. All compounds were compared with standards [29]. The compounds were characterized by their UV spectra and by a bracketed retention index. The compound called Epi had a chromophore similar to epifructigenine A = puberuline B [30], but this possible identification needs to be confirmed by mass spectrometry.

Results and discussion

Co-cultivation reveals interspecies competition

Species of *Aspergillus* normally grow in soil environments where they come in contact with various other microorganisms. Thus it is likely that they have evolved with mechanisms to deal with the constant competition. Each of the species of *Aspergillus* studied was grown in co-cultivation with other species to determine whether some were more adept at outcompeting adversaries. Equal numbers of spores from two species were plated on YDA and allowed to grow for 4 days. Spores were collected, and survivors were determined by diluting, plating, and identifying species by their colony color and morphology (See Fig. 1). The survivors are listed in Table 2. In cases where only one species is listed, the non-dominant species could not be detected at any dilution. These data show that the fitness of the species depends on the competitors, and not on the species itself. For example, *A. flavus* was able to outcompete *A. fumigatus* Af 293 (Afu 293), *A. niger* FGSC3122 (Ani 3122), and *N. fischeri* (Nfi), but was outgrown by *A. fumigatus* FGSC1163 (Afu 1163) and *A. niger* FGSC328 (Ani 328) (Table 2). In general, both strains of *A. fumigatus* and *A. terreus*

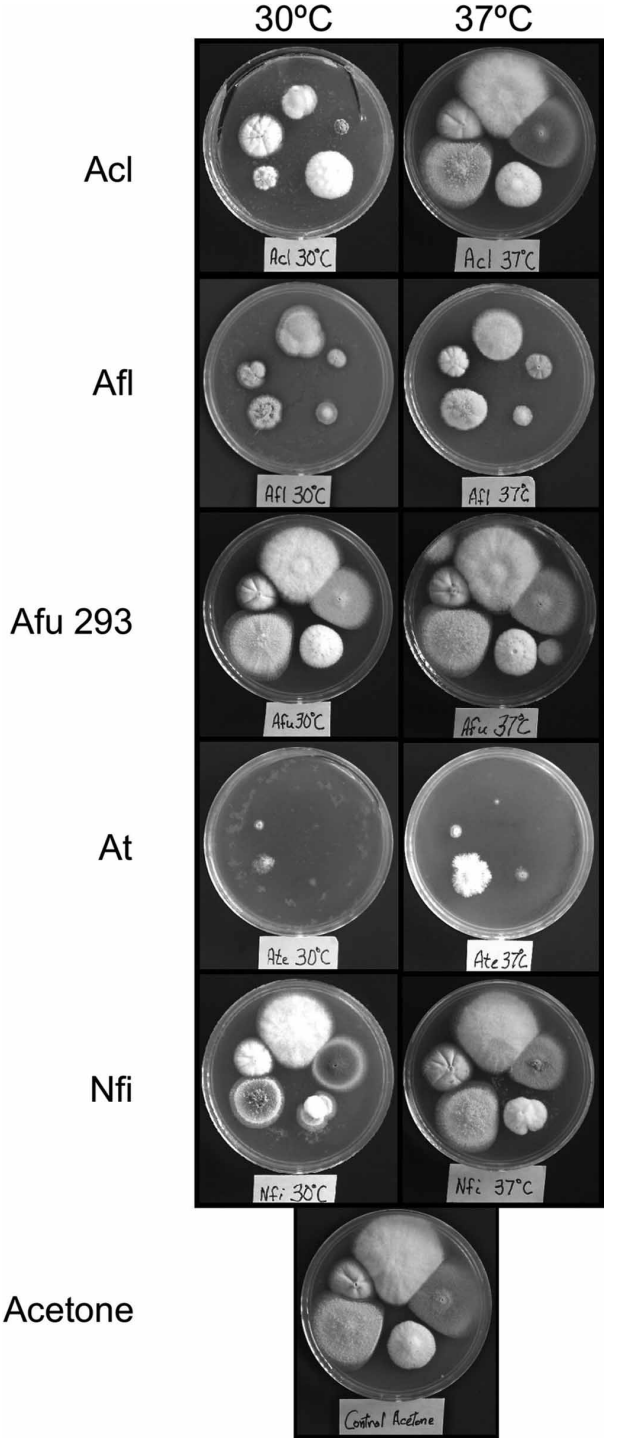

Fig. 1 Effect of mono-culture extracts on growth and conidiation of different species: The extracts from fungi grown in mono-culture at 30°C and 37°C were spread on a YDA plate and inocula of Nfi, Afu 293, Afl, Acl, and Aor (from top, clockwise) were grown on the extracts for 3 days. An acetone control is used as a comparison. This Figure is reproduced in colour in *Medical Mycology* online.

Table 2 Survivors from competition among species at 30°C. Species on the rows were grown for 4 days with competitor in the columns on YDA. Collected spores were diluted and plated to identify surviving species as described in Materials and Methods.

	Ani 3122	Ani 328	Nfi	Afu 293	Afu 1163	Afl	At	Aor	Acl	Anid
Ani 3122	N	Ani 328	Both	Both	Both	Afl	Both	Aor	Acl	Anid
Ani 328		N	Ani 328	Ani 328	Ani 328	Ani 328	Ani 328	Ani 328	Both	Anid
Nfi			N	Nfi	Nfi	Afl	Nfi	Aor	Acl	Anid
Afu 293				N	Both	Afl	At	Aor	Acl	Anid
Afu 1163					N	Afu 1163	Afu 1163	Aor	Acl	Anid
Afl						N	Afl	Aor	Acl	Anid
At							N	Aor	Acl	Anid
Aor								N	Acl	Anid
Acl									N	Anid
Anid										N

NIH2426 (At) were poor competitors, either having no effect or being outgrown by their competitors. In contrast, *A. clavatus* NRRL1 (Acl), both strains of *A. nidulans,* and *A. oryzae* RIB40 (Aor) appeared to be a good competitors, outgrowing almost all other species. Interestingly, Aor did not outcompete Ani 328, which underscores the importance of the specific strain of challenger instead of species fitness alone.

A particularly striking finding was the observation that *A. oryzae* could outcompete *A. flavus* in co-cultivation at 30°C. *A. flavus* is known for its superior adaptability in the environment, whereas *A. oryzae* is generally poor in its ability to adapt to harsh environments. This observation demonstrated that *A. oryzae* is better adapted to rich medium conditions (e.g., laboratory defined culture media) than is *A. flavus,* which is consistent with the fact that *A. oryzae* has been cultivated and selected under similar conditions for thousands of years for its use in fermentative processes. Secondly, selection for food flavor in fermentation may have preserved and enriched additional pathways for flavor-related compounds or secondary metabolites in *A. oryzae*. These compounds may have inadvertent antifungal activities.

A. oryzae extracts not only hindered the growth of *A. flavus*, but also altered the morphology of the Afl colonies such that they produced long and slim mycelia, which gave a fluffy appearance to the colony (data not shown). In some cases, Afl's normal green conidia became yellow–orange in color in the presence of different extracts (Fig. 1). The green spore color is a due to pigmentation with melanin or melanin-derivatives, which are also secondary metabolites. It is generally accepted that strains of *A. flavus* are variable in their morphology and aflatoxin production. For example, the aflatoxin producing ability is frequently lost after a few rounds of laboratory cultivation on the same medium. The observed change in conidial color and colony morphology in *A. flavus* could be due to the same mechanism as the loss of aflatoxin production in response to the environmental stresses, which in this case would be the secondary metabolites from another fungus.

It is not likely that the observed dominance patterns during co-cultivation are simply a matter of relative growth rates. For example, in almost every case of competition between Acl and another species, Acl was the only survivor after 72 h. However, in monoculture Acl grows at a rate of 0.66 cm/day compared to rates ranging from 0.77 cm/day for At, to 1.17 cm/day for Afl or Afu 293, and 1.33 cm/day for Nfi (Table S1 – online version only). In addition, Afl grew at the same rate as Afu 293 (1.16 cm/day), but only Afl was found in the co-cultivation plate. These data show that the order of relative growth-rate is independent of the pattern of co-cultivation dominance. Because differences in growth rates do not account for the observed dominant competitor, it is probable that Aspergilli use a different competitive mechanism, such as secretion of secondary metabolites.

Because some of these species can grow in a mammalian host [31], the same experiments were performed at 37°C for a subset of species to determine if they would be more effective competitors under those conditions. The temperature shift affected species differently. *A. clavatus* and *A. flavus* were negatively affected by the higher temperature as they were both unable to outcompete other species to the same extent as they had at 30°C (Table 3). In contrast, *A. fumigatus* and *A. terreus* were better competitors at 37°C than at 30°C, since they gained the ability to out compete more species (Table 3). These results are consistent with more than 90% of the cases of invasive asperigillosis being due to *A. fumigatus* [31]. *N. fischeri* did not appear affected by the change in temperature (Table 3).

Table 3 Survivors from competition among species at 37°C. Species on the rows were grown for 4 days with competitor in the columns on YDA. Collected spores were diluted and plated to identify surviving species as described in Materials and Methods.

	Nfi	Afu 293	Afl	At	Acl
Nfi	N	Both	Both	Nfi	Poor Growth
Afu 293		N	Both	Both	Afu 293
Afl			N	Afl	Afl
At				N	At
Acl					N

It is possible that each species produces variable secondary metabolites depending on external conditions and competition. That is, a species could sense the presence of a competitor and consequently increase or decrease the production of certain metabolites in an attempt to prevail over the competitor. For instance, the secondary metabolite farnesol from *C. albicans* was shown to have antagonistic effects against *A. nidulans* by specifically inducing apoptosis [32]. Alternatively, different species can have inherent resistances to metabolic compounds such that when they encounter competitors they are able to circumvent deleterious effects from the competitors' secondary metabolites. Genome-wide analyses of secondary metabolite clusters and corresponding resistance genes of several species of *Aspergillus* has revealed major differences in the presence of secondary metabolite biosynthetic genes [33]. These cases are not mutually exclusive, and it is likely that the differential growth and fitness observed in the presence of another species could be a result of a combination of variable secondary metabolite profiles and the presence of resistance genes.

Effect of monoculture extracts on growth and conidiation

One of the methods of competition used by fungi is the secretion of inhibitors that delay or abolish growth of other species. To test whether the competition effects observed in co-cultivation were due to secreted compounds, the culture extracts from each individual species was assayed for its effect on the growth or conidiation of other species. The extracts from each of the species had an impact on the growth or conidiation of the species tested. The Afu 293 extract had the least effect on the growth of any of the fungi on the test plates, but enhanced conidiation in many of those species (Table 4 and S1, and Fig. 1). Since conidiation was greater, it is likely that the extracts contained metabolites that could affect the tester species, either by directly inducing the cells into a different phase of

Table 4 Effect of mono- and co-culture extracts on growth of different species. Species in the columns were grown on YDA at 30°C in the presence of extracts acquired from cultures in the rows at the given temperatures. Growth effects were assessed as described in Materials and Methods.

	30°C					37°C				
	Afu 293	Nfi	Acl	Afl	At	Afu 293	Nfi	Acl	Afl	At
Acl	EC;DRG[1]	RG	C	EC;RG	SRG	EC;SRG	C	C	EC;C	SRG
Afl	DRG;EC	RG	RG	EC;RG	DRG	DRG;EC	EC;RG	DRG	EC;RG	RG
Afu 293	EC;SRG	EC;SRG	EC;RG	EC;C	C	SEC;SRG	SEC;C	SEC;C	SEC;C	SRG
At	DRG	DRG	DRG	DRG	DRG	DRG	DRG	DRG	RG	DRG
Nfi	EC;DRG	EC;SRG	EC;SRG	EC;RG	SRG	EC;RG	EC;C	EC;RG	EC;RG	SRG
Acl-Afl	DRG	DRG	RG	C	nt	nt	nt	nt	nt	nt
Acl-Afu 293	DRG	DRG	C	DRG	SRG	RG	SRG	RG	RG	DRG
Acl-At	RG	RG	RG	RG	RG	nt	nt	nt	nt	nt
Acl-Nfi	DRG	DRG	C	DRG	nt	nt	nt	nt	nt	nt
Afl-Afu 293	DRG	DRG	DRG	SRG	RG	RG;EC	RG;EC	EC;RG	RG	RG
Afl-At	SRG	SRG	SRG	SRG	SRG	SRG	SRG	SRG	SRG	SRG
Afl-Nfi	DRG	DRG	RG	EC;C	nt	EC;C	C	C	EC;C	C
Afu 293-At	DRG	DRG	RG	RG	RG	RG	DRG	DRG	RG	RG
Afu 293-Nfi	EC;RG	C	RG	EC;C	nt	EC;RG	EC;RG	RG	EC;C	nt
At-Nfi	SRG	SRG	SRG	SRG	SRG	SRG	SRG	SRG	SRG	SRG

[1]C, same as control; EC, Enhanced conidiation; SEC, slightly enhanced conidiation; DRG, dramatically reduced growth; RG, Reduced growth; SRG, slightly reduced growth; nt, not tested.

their growth cycle, or by interfering with proper growth signals, which could initiate a stress response. Indeed, conidiation in the filamentous fungus *Penicillium cyclopium* can be controlled by a secondary metabolite conidiogenone [34], and it has been proposed that conidiation of *A. nidulans* and *A. fumigatus* are controlled by diffusible signals also associated with secondary metabolism [35–37].

The extract from Afl reduced the growth of itself, Acl, and Nfi, and drastically reduced the growth of Afu 293 and At (Table 4 and S1, and Fig. 1). In addition, this extract enhanced the conidiation of Afl and Afu 293. In contrast, the extracts from At drastically reduced the growth of all species, including itself (Table 4 and S1, and Fig. 1), but no marked effect due to this extract was observed on conidiation. Aor was able to reduce the growth and enhanced the conidiation of only two species on the test plate, but had no effects on the other species tested (data not shown).

The range of inhibitory effects observed in these extracts is intriguing. For instance, almost every species tested had a different reaction to the Acl extract; some were slightly inhibited, some were severely diminished in growth rate, and others appeared to be undisturbed (Table 4 and S1, and Fig. 1). It is likely the extract contains compounds that are highly repressive, and different species have varying resistances, which supports the notion that the interaction between two species is a determinant of dominance. Also of interest is the ability of some extracts to inhibit even the growth of the specific species that produced it, as is the case of At (Table 4 and S1, and Fig. 1). This might be a situation in which the secreted compound induces expression of the resistance gene simultaneously, thus allowing the cells to grow while the concentration of the toxin increases. However, if the cells are presented with high levels of the compound, necessary resistance mechanisms have not been expressed and little or no growth occurs.

Co-cultivation modifies the biological activity and contents of culture extracts

Since co-cultivation revealed competition among species and it appeared that some species secreted inhibitors of growth, it was possible that growing two species together would result in the secretion of even greater quantities or a larger variety of inhibitors. To test this hypothesis, the extracts from co-cultivation plates were used to assess their effects on the growth and conidiation of other species. Several of the extracts appeared to have a different activity as a result of co-cultivation, and others did not seem to be modified in activity. The extract of the Afl monoculture reduced the growth of all other species, and none of the co-cultivation extracts had either more or less potency in their inhibition (Fig. 2 and Table 4 and S1). These results were consistent with Afl's ability to out compete all other species except Acl (see above). Analysis of the extracts using gas chromatography demonstrated that the chemical composition of the extracts was essentially the same when Afl was grown in mono-culture or in co-culture with Afu (Table 5).

Interestingly, a shift in temperature changed the composition of the extracts and decreased the inhibitory effect on the growth of other species (Tables 4 and 5). In most cases, a greater diversity of metabolites was found at 30°C. Furthermore, those extracts from 30°C had higher antifungal activities than did those extracts from cultures grown at 37°C. Other studies on the effect of a temperature shift from 30°C to 37°C indicated that

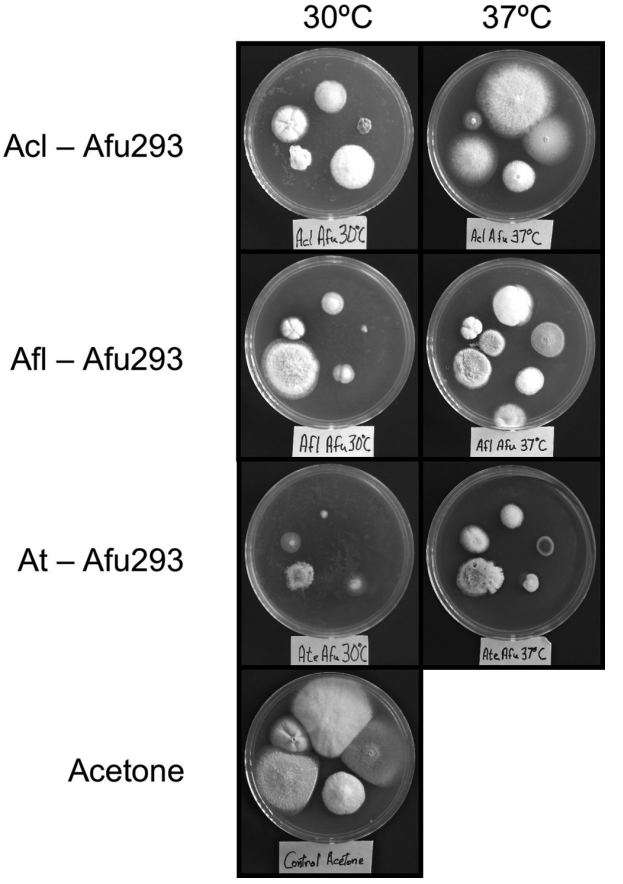

Fig. 2 Effect of co-cultivation extracts on growth and conidiation of different species: Acl, Afl, and At. The extracts from fungi grown in co-culture with Afu at 30°C (left) and 37°C (right) were spread on a YDA plate and inocula of Nfi, Afu, Afl, Acl, and At (from top, clockwise) were grown on the extracts for 3 days. An acetone control is used as a comparison (bottom). This Figure is reproduced in colour in *Medical Mycology* online.

Table 5 Metabolites detected with gas chromatography in mono- and co-culture extracts. Species in the columns were grown on YDA at given temperatures. Extracts acquired and analyzed by gas chromatography and compared with known standards [29]

Afl 30°C	Afl 37°C	Afu 293 30°C	Afu 293 37°C	Acl 30°C	Acl 37°C	Afl/Afu 293 30°C	Afl/Afu 293 37°C	Acl/Afu 293 30°C
Aflatoxin B1	Aflatoxin B1	Fumigaclavine C	Fumigaclavine C	Kotanin	Antafumicin	Kojic acid	Epi	Cyochalasin E
Aflatoxin B2	Aflatoxin B2	Pseurotin A	Pseurotin A	Orlandin	Cyochalasin E	Aflatoxin B1		Cytochalasin K
Oryzachlorin	Oryzachlorin	Pseurotin X	Pseurotin X		Cytochalasin K	Aflatoxin B2		
Cyclopiazonic Acid	Cyclopiazonic Acid	Gliotoxin	Gliotoxin		Kotanin	Oryzachlorin		
Epi	Epi	Fumiquinazoline 1	Fumiquinazoline 1			Cyclopiazonic Acid		
		Fumiquinazoline 2	Fumiquinazoline 2			Epi		
		Monomethylsulochrin	Monomethylsulochrin					
			Trypacidin					

the aflatoxin pathway genes are turned off at 37°C [38], possibly due to inactivation of the regulatory protein AflR [39]. Some of the secondary metabolite pathways may have been shut down at 37°C by a similar mechanism. It is also possible that at 37°C Afu can prevent or degrade any of Afl's secreted inhibitors at and in this fashion survive in co-cultivation with Afl. These inhibitors are not likely to be aflatoxin, since the synthesis of aflatoxin by Afl has been demonstrated to be inhibited at 37°C [38,39]. Also of note is that none of secondary metabolites of Afu are present in co-cultivation with Afl (Table 5). The absence of metabolites might be due to cross-regulation from one species to another as they vie for the same niche. The production of sterigmatocystin, a secondary metabolite in *A. nidulans,* has been associated with a diffusible signal in a fashion similar to the induction of conidiation [2].

Co-cultivation of Acl with Afu 293 or Nfi resulted in a greater inhibitory potency than any corresponding monoculture extracts (Table 4 and S1), despite the fact that Acl outcompeted Nfi, but not Afu 293. Gas chromatographic analysis of the Acl extract showed that co-cultivation modified the composition: kotanin and orlandin were absent, whereas cytochalasin E and K were present (Table 5). It is possible that cytochalasin E and K are part of a larger stress response, since these were also present in the extracts of Acl from growth at 37°C, a temperature at which the growth rate of Acl is greatly reduced. Cytochalasins may be responsible for the observed antifungal activity, since cytochalasins inhibit actin polymerization in eukaryotes [40].

In contrast, the co-cultivation extracts of At with all other species, resulted in a diminished inhibitory potency when compared to At grown by itself (Fig. 2 and Table 4 and S1). This might be because At was a poor competitor in co-culture and as a result only small amounts of its metabolites accumulated in the medium. As discussed above, this reduced potency might be a result of negative regulation of toxin production by secreted combative compounds from other species.

In summary, the current study characterized the interaction between close species relatives in the *Aspergillus* and *Neosartorya* genera and the impact of those interactions on the secondary metabolite profile of each strain. The results suggest that the outcome of an interaction has no predictable pattern, since it appears that each species has variable metabolic profiles that can be adjusted and modified by extracellular cues and by competing species. It was shown that some extracts have very high inhibitory and antifungal capacity, and that in some cases co-cultivation could make culture extracts much more potent in this respect. It is not clear

whether the antifungal activity is due to a single metabolite or to a combination of secreted metabolites, but the potential for novel therapeutic agents is very encouraging. We consider that using a co-cultivation method for inducing and identifying novel antifungal compounds is very promising and cost-effective. In addition, understanding the full complement of secondary metabolites produced by each species can also lead us to better understanding the potential virulence factors held by individual *Aspergillus* species.

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